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(54) Title: REDUCTION OF NICOTINE LEVELS IN TOBACCO

(57) Abstract

The present invention provides novel methods for producing plants with reduced nicotine levels. The claimed methods involve introducing into plants expression cassettes comprising nucleic acid segments from genes which encode enzymes in the nicotine biosynthetic pathway or which degrade nicotine. The nucleic acid segments are linked to a promoter sequence in either the sense or anti-sense direction.

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REDUCTION OF NICOTINE LEVELS IN TOBACCO

FIELD OF THE INVENTION

This invention relates generally to the use of recombinant DNA methods for genetically altering plants. In particular it relates to the generation of tobacco plants having greatly reduced levels of nicotine and various nicotine derived n-nitrosoamine compounds.

BACKGROUND OF THE INVENTION

The widespread use of cigarette tobacco in our society leads to more health problems than any other socially accepted practice. Cigarette smoking is not only the number one cause of cancer and emphysema, but also contributes greatly to a variety of cardiovascular disorders. Although the health consequences of cigarette smoking are now well understood, millions of people are unable to quit. The addictive properties of cigarettes are largely attributable to the presence of nicotine, one of the most addictive substances known. In addition to being addictive, nicotine is also the precursor for a large number of carcinogenic compounds present in tobacco and synthesized in the body. The elimination of nicotine from tobacco would greatly reduce both the addictive and carcinogenic properties of cigarettes.

Although many methods by which to reduce the nicotine content or the nicotine delivery of cigarette tobacco have been developed, all of them adversely affect the flavor of the tobacco and the quality of the smoking experience in some way. The currently available methods include reducing nicotine delivery with the use of filters, use of naturally occurring low nicotine strains of tobacco, and enzymatic or chemical degradation of nicotine *in vitro*.

Specialized filters such as the carbon filter were designed to reduce the amount of nicotine ingested by the smoker. Unfortunately, a large number of fragrance and flavor

compounds present in tobacco smoke are trapped as well. As a result, cigarettes employing these filters are substantially less palatable. Some cigarettes utilize filter assemblies which are more porous than others. These allow fresh air to be drawn in and mixed with the smoke as it is being inhaled, thereby decreasing the amount of nicotine and tar ingested per puff. Smokers, however, are not satisfied by the taste or quality of the smoke derived from such cigarettes and compensate by drawing more deeply on the cigarette, or by resorting to more drastic measures such as snipping part of the filter off. All cigarette filters, however, regardless of design, construction or chemical composition, are unable to specifically trap nicotine and consequently trap many compounds, some of which are essential for the flavor and aroma of the cigarette.

Some brands of cigarettes use tobacco obtained from cultivars bred to have lower nicotine contents. The problem with these strains of tobacco is that they exhibit a concomitant reduction in the levels of other alkaloid compounds and their related metabolites. Thus the tobacco from such plants produces cigarettes having relatively poor flavor. Additionally, cigarettes using tobacco from these strains are said to be low tar, low nicotine and are thus presumed to be safer to smoke than other cigarettes. However, there is some evidence which suggests these strains may have reduced alkaloid levels because of a greater than normal degree of conversion from alkaloid to carcinogenic N-nitrosoamines. For this reason, tobacco cultivated from these strains may be more harmful than believed.

Several processes have been developed to reduce the nicotine content of the tobacco after it has been harvested. These processes primarily involve microbial enzymatic degradation, chemical treatment or high pressure extraction (see, e.g., U.S. Patents 4,557,280; 4,561,452; 4,848,373; 4,183,364; 4,215,706, all of which are incorporated herein by reference).

SUMMARY OF THE INVENTION

The present invention provides methods for reducing nicotine levels in plants, usually a member of the genus Nicotiana, using recombinant DNA techniques. The methods involve introducing into the plant an expression cassette comprising a promoter sequence operably linked to a nucleic acid segment from a gene encoding an enzyme in the nicotine biosynthetic pathway. The nucleic acid segment may be linked to the promoter in the anti-sense or sense orientation. Alternatively, the expression cassette may comprise a nucleic acid segment from a gene encoding a nicotine degradative enzyme.

Typically, the biosynthetic enzyme is nicotine synthase or methyl putrescine oxidase and the degradative enzyme is nicotine demethylase. The expression cassette can be introduced into the plant using in vitro techniques or by a sexual cross.

The invention also provides plants comprising a heterologous expression cassette comprising a promoter sequence operably linked to a nucleic acid segment from a gene encoding an enzyme in the nicotine biosynthetic pathway. The nucleic acid segment may be linked to the promoter in the anti-sense or sense orientation.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the nicotine biosynthetic pathway.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The biosynthesis of nicotine in tobacco plants has been extensively examined and proceeds via the pathway shown in the Figure 1. See, e.g., Leete, in Alkaloids: Chemical and Biological Perspectives, W. Pelletier, ed., pp. 85-151 (Wiley Interscience, 1983) and Friesen et al., Tet. Lett. 31:6295-6298 (1990), both of which are incorporated herein by reference. Nicotine is formed as the result of a bifurcated pathway, the arms of which can be seen to originate from ornithine and nicotinic acid.

Ornithine is first decarboxylated by the enzyme ornithine decarboxylase (3) to yield putrescine. Putrescine is

then methylated by the enzyme putrescine methyl transferase (4) to give n-methyl putrescine which is then oxidized by n-methyl putrescine oxidase (5) to give n-methyl pyrroline.

Nicotinic acid is enzymatically reduced (1), decarboxylated (2), and finally condensed with n-methyl pyrroline to produce nicotine. The condensation reaction is carried out by the enzyme nicotine synthase (6). Nicotine is degraded in the leaves by the enzyme nicotine demethylase (7).

The present invention provides methods for disrupting the biosynthesis of nicotine in plants that synthesize nicotine, in particular, members of the genus Nicotiana by blocking any of the enzymatic steps shown in Figure 1. Of particular interest are the following enzymes: nicotine reductase (1), nicotine decarboxylase (2), ornithine decarboxylase (3), putrescine methyl transferase (4), methyl putrescine oxidase (5), and nicotine synthase (6).

Any of these steps can be blocked using, for instance, anti-sense regulation of the gene associated with that step. To accomplish this, a nucleic acid segment from the desired gene is cloned and operably linked to a promoter such that the anti-sense strand of RNA will be transcribed. The construct is then transformed into plants and the anti-sense strand of RNA is produced. In plant cells, it has been shown that anti-sense RNA inhibits gene expression by preventing the accumulation of mRNA which encodes the enzyme of interest, see, e.g., Sheehy et al., Proc. Nat. Acad. Sci., 85:8805-8809 (1988), and Hiatt et al., U.S. Patent No. 4,801,340 which are incorporated herein by reference.

Introduction of cloned nucleic acid segments configured such that the sense-strand of RNA is produced, is also an effective means by which to block the transcription of the nicotine biosynthetic genes. For an example of the use of this method to modulate expression of endogenous genes see, Napoli et al., The Plant Cell 2:279-289 (1990), and U.S. Patent No. 5,034,323, which are incorporated herein by reference.

Another means by which to generate nicotine free tobacco plants is through the use of degradative enzymes. A number of different degradation enzymes are available and can

be used for this purpose. For instance, Figure 1 shows the degradation of nicotine to nornicotine via demethylation. The reaction is carried out, at least in part, by the enzyme nicotine demethylase (7) in tobacco leaves.

Nicotine is synthesized primarily in the roots and then transported to the stems and leaves where it accumulates. In the latter stages of the plant's life cycle nicotine demethylase is expressed in the leaves where it degrades nicotine. In order to generate plants with low levels of nicotine, nucleic acid segments from the nicotine demethylase gene, can be cloned and put under the control of a root specific promoter, such as those described in Benfey, EMBOJ 81:2195-2202 (1989) and Jorgensen et al., Nucleic Acids Res., 16:39(1988) which are incorporated herein by reference.

A number of bacterial strains have been shown to degrade nicotine. For instance, a crude protein extract from Arthrobacter was applied to tobacco leaves and found to degrade nicotine in vitro. The enzyme nicotine demethylase is isolated from tobacco leaf extracts using conventional chromatographic procedures such as gel filtration, ion exchange, and ligand affinity chromatography according to standard techniques. The N-terminal amino acid sequence is determined and oligonucleotide probes designed to hybridize to its corresponding DNA sequence are synthesized. These probes are then used to screen tobacco cDNA libraries. Alternatively, the presence of restriction fragment length polymorphisms (RFLP) between wild type and mutant varieties of tobacco can be used to identify the chromosomal location of the demethylase gene. Genetic evidence indicates that the demethylation of nicotine is heritable as a single dominant gene. The gene encoding the degradative enzyme is then conveniently introduced into plants, where it is expressed in vivo.

I. General Methods

Generally, the nomenclature used hereafter and the laboratory procedures in recombinant DNA technology described below are those well known and commonly employed in the art. Standard techniques are used for cloning, DNA and RNA

isolation, amplification and purification. Generally enzymatic reactions involving DNA ligase, DNA polymerase, restriction endonucleases and the like are performed according to the manufacturer's specifications. These techniques and various other techniques are generally performed according to Sambrook et al., Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, (1989), which is incorporated herein by reference.

The steps required to clone and subsequently block any of the nicotine biosynthetic genes are well known to one of skill in the art. In summary, the manipulations necessary to prepare nucleic acid segments suitable for use in the present invention and introduce them into a plant cell involve 1) purifying desired enzymes from the appropriate sources (e.g., bacterial or plant), 2) preparing oligonucleotide probes corresponding to a portion of the amino acid sequence of the purified enzymes, 3) screening a cDNA or genomic library for the sequences which hybridize to the probes, 4) linking a plant promoter and other sequences necessary for expression to the identified sequences (the sequences can be linked in the anti-sense or sense direction), 5) transforming suitable host plant cells, and 6) selecting and regenerating cells which transcribe the sequences and display reduced nicotine levels.

II. Purification of Enzymes and Synthesis of Oligonucleotide Probes

Methods for purifying desired proteins are well known in the art and are not presented in detail here. For a review of standard techniques see, Methods in Enzymology, "Guide to Protein Purification", M. Deutscher, ed. Vol. 182 (1990), which is incorporated herein by reference. For instance, methyl putrescine oxidase (MPO) is purified according to the methods described in Mizusaki et al., Phytochemistry 11:2757, which is incorporated herein by reference. The enzyme MPO is isolated from tobacco root extracts by passing homogenized root tissue over a PD 10 gel filtration column to remove phenolic compounds. Further purification of the extract typically includes conventional chromatographic procedures such as ion

exchange, dye affinity, hydroxyl apatite, and ligand affinity chromatography. It is typically purified to the point that it can be identified as a distinct band by polyacrylamide gel electrophoresis (PAGE). Passage over two or three chromatography columns is sufficient as the enzyme need not be purified to homogeneity prior to PAGE.

The enzyme is then assayed in the gel matrix for its activity. For example, MPO activity is assayed using N-methyl putrescine as a substrate and detecting the reaction product, n-methyl-delta-pyrrolinium chloride, at 210 nm upon separation and elution from reactants using HPLC cation exchange column chromatography at pH3.5 as described in Feth et al., Phytochemistry 24:1653-1655 (1985), which is incorporated herein by reference. MPO is also assayed by separation and detection of its reaction product using thin layer chromatography (TLC) according to standard procedures.

Purification of the enzyme nicotine synthase requires both substrates of the enzyme, the methyl-pyrrolinium salt and the decarboxylated, reduced intermediate derived from nicotine acid. A procedure for the synthesis of the methyl-pyrrolinium salt has been published (Leete et al., Phytochemistry 27:401-406 (1988), which is incorporated herein by reference).

Alternatively, both of these compounds are easily synthesized in vitro using crude tobacco root extracts. A suitable method for obtaining these compounds is as follows. Putrescine and carbon 14 radiolabeled s-adenosyl methionine (the methyl donor in this reaction) is added to a crude tobacco root extract and incubated under conditions favorable for the enzymatic conversion of putrescine to methyl-putrescine and its subsequent conversion to the methyl-pyrrolinium salt (see, Leete et al and Feth et al, supra). The reaction conditions for these two reactions are sufficiently close that both enzymatic steps will occur in the same buffer (50mM NaPO4, pH 8.0). The starting materials are added in excess (e.g., at least 1000-fold) of the substrates in the extract. Since the reaction mixture lacks appreciable amounts of nicotinic acid, the methyl-pyrrolinium salt accumulates. The reaction product

is then partially purified using a cationic exchange column which will bind the positively charged methyl-pyrroline.

The decarboxylated, reduced form of nicotinic acid is synthesized in vitro by a similar procedure. Nicotinic acid is added to crude tobacco root preparations and incubated under conditions which have been demonstrated to favor the conversion of nicotinic acid and the methyl-pyrrolinium salt to nicotine (see, Leete, in Alkaloids: Chemical and Biological Perspectives, supra). In this case, since the methyl-pyrrolinium salt is not added, the reaction terminates at the step prior to the condensation reaction and the desired compound would be accumulated. This compound can be partially purified using, for instance, hydrophobic chromatography. The compound will separate from proteins and more polar species on a reversed phase C4 column.

Having both of these preparations one can assay protein fractions under the conditions known to be favorable for the nicotine synthase reaction. In this reaction the carbon 14 radiolabeled methyl-pyrrolinium salt will condense with the reduced, decarboxylated derivative of nicotinic acid and radiolabeled nicotine will be formed. The reaction products can be easily separated using TLC and HPLC (see, Friesen et al., supra) and detected using, for instance, autoradiography. The enzyme is then purified according to standard procedures as described above.

After isolation of the enzyme, the amino acid sequence of the N-terminus is determined and an oligonucleotide probe, designed to hybridize to the desired gene, is synthesized. Amino acid sequencing is performed according to standard techniques (see, e.g., Matsudaira J. Biol. Chem. 262:10035-10038 (1987), which is incorporated herein by reference. Oligonucleotide probes are synthesized according to standard techniques as described, for instance, in Sambrook et al., supra.

Oligonucleotide probes useful for identification of desired genes can also be prepared from conserved regions of related genes in other species. For instance, probes derived from a gene encoding a transferase from related species may be

used to screen libraries for putrescine methyl transferase. Alternatively, the presence of restriction fragment length polymorphisms (RFLP) between wild type and mutant varieties of the plant can be used to identify the desired gene.

III. Preparation of Libraries for Screening

Genomic or cDNA libraries are prepared according to standard techniques as described, for instance, in Sambrook, supra. To construct genomic libraries, large segments of genomic DNA are generated by random fragmentation and are ligated with vector DNA to form concatemers that can be packaged into the appropriate vector. Two kinds of vectors are commonly used for this purpose, bacteriophage lambda vectors and cosmids.

In the present invention, cDNA libraries are generally used to screen for the desired gene. To prepare cDNA from various genes in the nicotine biosynthetic pathway, mRNA from tobacco roots is first isolated. Eukaryotic mRNA has at its 3' end a string of adenine nucleotide residues known as the poly-A tail. Short chains of oligo d-T nucleotides are then hybridized with the poly-A tails and serve as a primer for the enzyme, reverse transcriptase. This enzyme uses RNA as a template to synthesize a complementary DNA (cDNA) strand. A second DNA strand is then synthesized using the first cDNA strand as a template. Linkers are added to the double-stranded cDNA for insertion into a plasmid or λ phage vector for propagation in E. coli.

Identification of clones harboring the desired nucleic acid segments is performed by either nucleic acid hybridization or immunological detection of the encoded protein, if an expression vector is used. The bacterial colonies are then replica plated on nitrocellulose filters. The cells are lysed and probed with either oligonucleotide probes described above or with antibodies to the desired protein.

IV. Vector Construction

Selection of an appropriate vector useful in the present invention is relatively simple, as the constraints are minimal. The minimal requirements of the vector are that the desired nucleic acid sequence be introduced in a relatively intact state. Thus, any vector which will produce a plant carrying the introduced DNA sequence should be sufficient. Any vector which will introduce a substantially intact RNA which can ultimately be converted into a stably maintained DNA sequence is also acceptable.

Even a naked piece of DNA would be expected to be able to confer the properties of this invention, though at low efficiency. The decision as to whether to use a vector, or which vector to use, will be guided by the method of transformation selected. This determination is considered to be well with in the ordinary skill of those in the art.

The vectors useful in the present invention include, but are not limited to, the Ti plasmid vectors, shuttle vectors designed to maximally yield high numbers of copies, episomal vectors containing minimal sequences necessary for ultimate replication once transformation has occurred, and viral vectors, including the possibility of RNA forms of the gene sequences. The selection of vectors and methods to construct them are commonly known to persons of ordinary skill in the art and are described in general technical references (See, in general, Methods in Enzymology Vol. 153 ("Recombinant DNA Part D") 1987, Wu and Grossman Eds., Academic Press, incorporated herein by reference). The vectors typically comprise additional attached sequences which confer resistance to degradation of the nucleic acid fragment, which assist in the process of genomic integration, or which provide a means to easily select for those cells or plants which are transformed. Such sequences are advantageous and greatly decrease the difficulty of selecting useable transformed plants.

The recombinant vectors of the present invention typically comprise an expression cassette designed for initiating transcription of the desired nucleic acid segments in plants. Companion sequences, of bacterial or viral origin,

are also included to allow the vector to be cloned in a bacterial or phage host. The vector will preferably contain a broad host range prokaryote origin of replication. A selectable marker should also be included to allow selection of bacterial cells bearing the desired construct. Suitable prokaryotic selectable markers include resistance to antibiotics such as kanamycin or tetracycline.

Other DNA sequences encoding additional functions may also be present in the vector, as is known in the art. For instance, in the case of Agrobacterium transformations, T-DNA sequences will also be included for subsequent transfer to plant chromosomes.

For expression in plants, the recombinant expression cassette will contain, in addition to the desired nucleic acid segment, a plant promoter region, a transcription initiation site (if the sequence to be transcribed lacks one), and a transcription termination sequence. Unique restriction enzyme sites at the 5' and 3' ends of the cassette are typically included to allow for easy insertion into a pre-existing vector.

Sequences controlling eukaryotic gene expression have been extensively studied. Promoter sequence elements include the TATA box consensus sequence (TATAAT), which is usually 20 to 30 base pairs (bp) upstream of the transcription start site. In most instances the TATA box is required for accurate transcription initiation. By convention, the transcription start site is called +1. Sequences extending in the 5' (upstream) direction are given negative numbers and sequences extending in the 3' (downstream) direction are given positive numbers.

In plants, further upstream from the TATA box, at positions -80 to -100, there is typically a promoter element with a series of adenines surrounding the trinucleotide G (or T) N G. J. Messing et al., in Genetic Engineering in Plants, pp. 221-227 (Kosage, Meredith and Hollaender, eds. 1983), which is incorporated herein by reference. Other sequences conferring tissue specificity, response to environmental signals, or maximum efficiency of transcription

may also be found in the promoter region. Such sequences are often found within 400 bp of transcription start site, but may extend as far as 2000 bp or more.

In the construction of heterologous promoter/structural gene combinations, the promoter is preferably positioned about the same distance from the heterologous transcription start site as it is from the transcription start site in its natural setting. As is known in the art, however, some variation in this distance can be accommodated without loss of promoter function.

The particular promoter used in the expression cassette is a noncritical aspect of the invention. Any of a number of promoters which direct transcription in plant cells is suitable. The promoter can be either constitutive or inducible. Promoters of bacterial origin include the octopine synthase promoter, the nopaline synthase promoter and other promoters dérived from native Ti plasmids. Herrara-Estrella et al., Nature, 303:209-213, 1983. Viral promoters include the 35S and 19S RNA promoters of cauliflower mosaic virus. Odell et al. Nature, 313:810-812, 1985. Possible plant promoters include the ribulose-1,3-bisphosphate carboxylase small subunit promoter and the phaseolin promoter.

In addition to a promoter sequence, the expression cassette should also contain a transcription termination region downstream of the structural gene to provide for efficient termination. The termination region may be obtained from the same gene as the promoter sequence or may be obtained from different genes.

If the mRNA encoded by the structural gene is to be efficiently translated, polyadenylation sequences are also commonly added to the vector construct. Alber and Kawasaki, Mol. and Appl. Genet, 1:419-434, 1982. Polyadenylation sequences include, but are not limited to the Agrobacterium octopine synthase signal (Gielen et al., EMBO J., 3:835-846, 1984) or the nopaline synthase signal (Depicker et al., Mol. and Appl. Genet, 1:561-573, 1982).

The vector will also typically contain a selectable marker gene by which transformed plant cells can be identified

in culture. Usually, the marker gene will encode antibiotic resistance. These markers include resistance to G418, hygromycin, bleomycin, kanamycin, and gentamicin. After transforming the plant cells, those cells having the vector will be identified by their ability to grow in a medium containing the particular antibiotic.

Other features of the vectors of the present invention include various 5' untranslated leader sequences such as the "cab leader" from petunia or the "omega leader" from tobacco mosaic virus.

The nucleic acid segment to be introduced generally will be substantially homologous to the endogenous sequence intended to be repressed. As used herein "homologous" means corresponding to (the same as). RNA which is homologous to a gene, is RNA which corresponds to the template sequence (with the normal exception of uracil for RNA in place of thymidine for DNA). Thus, cellularly produced "homologous RNA", as used herein, is not complementary to the template DNA strand of the gene. The minimal homology of the introduced sequence will typically be greater than about 65%, but a higher homology will usually exert a more effective repression of expression of the endogenous sequences. Substantially greater homology, or more than about 80% is preferred, though about 95% to absolute identity would be most preferred. Consequently, the effect should apply to any other proteins within a similar family of genes exhibiting homology or substantial homology. For example, MPO may be encoded by one or more homologous genes which comprise a gene family. Thus, repression of one member of the family will typically serve to impose the same repressive effect on others of the family. Similarly, for example, MPO genes from other plant species may be utilized.

The term "substantial homology" as used herein refers to nucleotide sequences which share a majority of their sequence. Generally, this will be at least about 65% of their sequences and preferably about 95% of their sequence. Another indication that sequences are substantially homologous is if they hybridize under stringent conditions. Stringent conditions will depend upon various parameters and will be

different in different circumstances. Generally, stringent conditions are those in which the salt concentration is at least about 0.2 molar and the temperature is at least about 60°C.

The introduced sequence, needing less than absolute homology, also need not be full length, relative to either the primary transcription product or fully processed mRNA. A higher homology in a shorter than full length sequence compensates for a longer less homologous sequence. Furthermore, the introduced sequence need not have the same intron or exon pattern, and homology of non-coding segments will be equally effective. Normally, a sequence of between about 10 nucleotides and 1000 nucleotides should be used, though a sequence of between about 100 and about 1000 nucleotides is preferred, and a sequence of between about 500 and about 750 nucleotides is especially preferred.

It should be noted that since a full length coding sequence is unnecessary, it is possible to produce the same effect on multiple proteins using a single transformation by fusing multiple sequences together to coordinately repress various different genes. In addition, assuming a sufficient number of introductions are made, the introduced sequence need not be linked to an operative promoter sequence. However, as discussed above, use of a promoter sequence is preferred. Typically, partially or fully constitutive promoters (e.g., the cauliflower mosaic virus promoter) are used.

Where the vector comprises the nucleic acid segment in the sense orientation, the same effect is produced by the introduction of a strong promoter operably linked to the coding strand of an endogenous sequence. This method is useful for increasing expression of the targeted gene (e.g. nicotine demethylase). This can be effected by either the introduction of a promoter alone to a site operably linked to the target sequence (e.g., by homologous recombination), or by the reintroduction of a sequence of endogenous origin recombinantly attached to an operably linked promoter (resulting in a chimeric gene).

V. Transcription of the desired nucleic acid segment in plant cells

A. Transformation of plant cells by in vitro techniques

1. Direct Transformation

The vectors described above can be microinjected directly into plant cells by use of micropipettes to mechanically transfer the recombinant DNA. Crossway, Mol. Gen. Genetics, 202:179-185 (1985), which is incorporated herein by reference. The genetic material may also be transferred into the plant cell using polyethylene glycol, Krens, et al., Nature, 296, 72-74, (1982), which is incorporated herein by reference.

Another method of introduction of nucleic acid segments is high velocity ballistic penetration by small particles with the nucleic acid either within the matrix of small beads or particles, or on the surface, Klein, et al., Nature, 327, 70-73 (1987), which is incorporated herein by reference.

Yet another method of introduction is fusion of protoplasts with other entities, either minicells, cells, lysosomes or other fusible lipid-surfaced bodies, Fraley, et al., Proc. Natl. Acad. Sci. USA, 79, 1859-1863 (1982), which is incorporated herein by reference.

The DNA may also be introduced into the plant cells by electroporation. Fromm et al., Pro. Natl Acad. Sci. USA, 82:5824 (1985), which is incorporated herein by reference. In this technique, plant protoplasts are electroporated in the presence of plasmids containing the expression cassette. Electrical impulses of high field strength reversibly permeabilize biomembranes allowing the introduction of the plasmids. Electroporated plant protoplasts reform the cell wall, divide, and regenerate.

2. Vectored Transformation

Cauliflower mosaic virus (CaMV) may be used as a vector for introducing the anti-sense DNA into plant cells. (Hohn et al., 1982 "Molecular Biology of Plant Tumors," Academic Press, New York, pp.549-560; Howell, United States Patent No. 4,407,956), which are incorporated herein by

reference. In accordance with the described method, the entire CaMV viral DNA genome is inserted into a parent bacterial plasmid creating a recombinant DNA molecule which can be propagated in bacteria. After cloning, the recombinant plasmid is further modified by introduction of the desired sequence into unique restriction sites in the viral portion of the plasmid. The modified viral portion of the recombinant plasmid is then excised from the parent bacterial plasmid, and used to inoculate the plant cells or plants.

A preferred method of introducing the DNA into plant cells is to infect a plant cell with Agrobacterium tumefaciens or A. rhizogenes previously transformed with the gene. Under appropriate conditions known in the art, the transformed plant cells are grown to form shoots or roots, and develop further into plants.

Agrobacterium is a genus in the gram-negative family Rhizobiaceae. Its species are responsible for crown gall (A. tumefaciens) and hairy root disease (A. rhizogenes). The plant cells in crown gall tumors and hairy roots are induced to produce amino acid derivatives known as opines, which are catabolized only by the bacteria. The bacterial genes responsible for expression of opines are a convenient source of control elements for chimeric expression cassettes. In addition, assaying for the presence of opines can be used to identify transformed tissue.

Heterologous genetic sequences can be introduced into appropriate plant cells, by means of the Ti plasmid of A. tumefaciens or the Ri plasmid of A. rhizogenes. The Ti or Ri plasmid is transmitted to plant cells on infection by Agrobacterium and is stably integrated into the plant genome. J. Schell, Science, 237: 1176-1183 (1987), which is incorporated herein by reference.

Ti and Ri plasmids contain two regions essential for the production of transformed cells. One of these, named transferred DNA (T-DNA), is transferred to plant nuclei and induces tumor or root formation. The other, termed the virulence (vir) region, is essential for the transfer of the T-DNA but is not itself transferred. The T-DNA will be

transferred into a plant cell even if the vir region is on a different plasmid, such vectors are typically termed binary vectors. Hoekema, et al., Nature, 303:179-189 (1983), which is incorporated herein by reference. The transferred DNA region, can be increased in size by the insertion of heterologous DNA without its ability to be transferred being affected. A modified Ti or Ri plasmid, in which the disease-causing genes have been deleted, can be used as a vector for the transfer of the gene constructs of this invention into an appropriate plant cell.

Construction of recombinant Ti and Ri plasmids in general follows methods typically used with the more common bacterial vectors, such as pBR322. Additional use can be made of accessory genetic elements sometimes found with the native plasmids and sometimes constructed from foreign sequences. These may include but are not limited to "shuttle vectors", (Ruvkun and Ausubel, 1981, Nature 298:85-88), promoters, (Lawton et al., 1987, Plant Mol. Biol. 9:315-324) and structural genes for antibiotic resistance as a selection factor (Fraley et al., Proc. Nat. Acad. Sci., 80:4803-4807, 1983), all of which are incorporated herein by reference.

All plant cells which can be transformed by Agrobacterium and from which whole plants can be regenerated can be transformed according to the present invention to produce transformed intact plants which contain the desired DNA. There are two common ways to transform plant cells with Agrobacterium:

(1) co-cultivation of Agrobacterium with cultured isolated protoplasts, or

(2) transformation of intact cells or tissues with Agrobacterium.

Method (1) requires an established culture system that allows for culturing protoplasts and subsequent plant regeneration from cultured protoplasts.

Method (2) requires (a) that the intact plant tissues, such as cotyledons, can be transformed by Agrobacterium and (b) that the transformed cells or tissues can be induced to regenerate into whole plants.

Most dicot species can be transformed by Agrobacterium. All species which are a natural plant host for Agrobacterium are transformable in vitro. Monocotyledonous plants, and in particular, cereals, are not natural hosts to Agrobacterium. There is growing evidence now that certain monocots can be transformed by Agrobacterium. Using novel experimental approaches cereal species such as rye (de la Pena et al., Nature 325:274-276, 1987), corn (Rhodes et al., Science 240:204-207, 1988), and rice (Shimamoto et al., Nature 338:274-276, 1989) may now be transformed, see also, Hooykas-Van Slogteren et al., Nature, 311:763-764 (1984), all of which are incorporated herein by reference.

B. Selection and Regeneration of transformed plant cells

After transformation, transformed plant cells or plants comprising the anti-sense DNA must be identified. A selectable marker, such as those discussed, supra, is typically used. Transformed plant cells can be selected by growing the cells on growth medium containing the appropriate antibiotic. The presence of opines can also be used if the plants are transformed with Agrobacterium.

After selecting the transformed cells, one can confirm expression of the desired heterologous gene. Simple detection of mRNA encoded by the inserted DNA can be achieved by well known methods in the art, such as Northern blot hybridization. The inserted sequence can be identified by Southern blot hybridization, as well. See, e.g., Sambrook, supra.

After determination of the presence of the DNA, whole plant regeneration is desired. All plants from which protoplasts can be isolated and cultured to give whole regenerated plants can be transformed by the present invention. Some suitable plants include, for example, species from the genera Fragaria, Lotus, Medicago, Onobrychis, Trifolium, Trigonella, Vigna, Citrus, Linum, Geranium, Manihot, Daucus, Arabidopsis, Brassica, Raphanus, Sinapis, Atropa, Capsicum, Datura, Hyoscyamus, Lycopersicon, Nicotiana, Solanum, Petunia, Digitalis, Majorana, Cichorium, Helianthus, Lactuca, Bromus,

Asparagus, Antirrhinum, Hererocallis, Nemesia, Pelargonium, Panicum, Pennisetum, Ranunculus, Senecio, Salpiglossis, Cucumis, Browaalia, Glycine, Lolium, Zea, Triticum, Sorghum, Malus, Apium, and Datura. As discussed above, plants which synthesize nicotine, such Nicotiana, are particularly preferred. Other plants which synthesize nicotine and related alkaloids include members of the following genera Acacia, Datura, Cannabis, Equisetum, Lycopodium, Petunia, Solanum, Sedum, and Atropa.

Plant regeneration from cultured protoplasts is described in Evans et al., Handbook of Plant Cell Cultures, Vol. 1: (MacMillan Publishing Co. New York, 1983); and Vasil I.R. (ed.), Cell Culture and Somatic Cell Genetics of Plants, Acad. Press, Orlando, Vol. I, 1984, and Vol. III, 1986, which are incorporated herein by reference.

Means for regeneration vary from species to species of plants, but generally a suspension of transformed protoplasts or a petri plate containing transformed explants is first provided. Callus tissue is formed and shoots may be induced from callus and subsequently rooted. Alternatively, embryo formation can be induced in the callus tissue. These embryos germinate as natural embryos to form plants. The culture media will generally contain various amino acids and hormones, such as auxin and cytokinins. Efficient regeneration will depend on the medium, on the genotype, and on the history of the culture. If these three variables are controlled, then regeneration is usually reproducible and repeatable.

Regenerated plants with the desired characteristics are typically identified by determining levels of nicotine in the leaves. Typically this is done by extracting the nicotine from the leaves and determining the concentration by HPLC according to the method of Friesen, supra.

While many of these improvements suggested above are not essential, the efficiency of production of useful transformants may be significantly affected. Some of the transformants may be identical to the parental plants, others may have reduced nicotine levels in the leaves or other organs of interest. Others may have reduced nicotine levels in

certain cells or patches of cells or segments of leaves or other organs resulting in regular or irregular patterns. Leaves on the same plant may even have different patterns. The likelihood of obtaining a desirable transformant will depend upon the number of plants screened and the efficiency of actual transformation and expression of the foreign nucleic acid sequence.

In addition, a number of factors may affect expression of the introduced nucleic acid segment, including the type of promoter, the temporal pattern of the promoter, and the operation of the promoter in view of its position within the genome. A promoter which is expressed concurrently with or prior to the normal activation of the homologous endogenous sequence is preferred. A constitutive promoter is most preferred, such as the cauliflower mosaic virus promoter. This promoter is constitutive because its operation is relatively independent of the developmental stage of the cell in which it is contained. A regulated or inducible promoter, such as ones associated with the ribulose-1,5-bisphosphate carboxylase, the chlorophyll binding proteins or the glycine-rich root protein genes are also suitable. This control may be either temporal with respect to the developmental stage of the cell, or based upon differential expression by different parts or organs of the plant. The operation of a promoter may vary depending on its location in the genome. Thus, an inducible promoter may operate differently from how it does in its normal location, e.g., it may become fully or partially constitutive.

If the introduced nucleic acid segment is an intact gene from the target plant or other plant species (meaning a complete gene containing coding sequences, intron, promoter, enhancers and other cis-acting regulatory elements either upstream (5') or downstream (3') of the coding sequences), a fraction of independent transformed plants, may carry the introduced gene in locations that result in abnormal expression, i.e., expression at abnormal times in development. If the introduced gene is a chimeric gene (meaning that one or more elements, such as a promoter, from another gene has been substituted for a component of the intact gene or added to the

intact gene, including coding sequences fused to upstream and downstream sequences necessary or beneficial for expression) and is driven by a constitutive (fully or partially) promoter, then abnormal levels and times of expression will be achieved in a large fraction of the transformed plants. If the introduced gene is a chimeric gene and is driven by a developmentally regulated promoter, depending on the promoter, some fraction of plants will show abnormal levels and times of expression of the introduced gene. The strength of the promoter or other cis element can be the same, lower, or higher than the coding sequence's usual promoter. The timing in development can be earlier or the same.

Finally, one of skill will recognize that after the expression cassette is stably incorporated in transgenic plants and confirmed to be operable, it can be introduced into other plants by sexual crossing. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed.

Definitions

The phrases "nucleic acid sequence" or "nucleic acid segment" refer to a single or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end. It includes both self-replicating plasmids, infectious polymers of DNA or RNA and non-functional DNA or RNA.

The term "promoter" refers to a region of DNA upstream from the structural gene and involved in recognition and binding RNA polymerase and other proteins to initiate transcription. A "plant promoter" is a promoter capable of initiating transcription in plant cells.

The term "plant" includes whole plants, plant organs (e.g., leaves, stems, roots, etc.), seeds and plant cells. The class of plants which can be used in the method of the invention is generally as broad as the class of higher plants amenable to transformation techniques, including both monocotyledonous and dicotyledonous plants. It includes plants of a variety of ploidy levels, including polyploid, diploid and haploid. Plants from the genus Nicotiana are preferably used.

The phrase "suitable host" refers to a microorganism or cell that is compatible with a recombinant plasmid, DNA sequence or recombinant expression cassette and will permit the plasmid to replicate, to be incorporated into its genome, or to be expressed.

The term "expression" refers to the transcription and translation of a structural gene so that a protein is synthesized.

A "constitutive" promoter is a promoter which is active under most environmental conditions and states of development or cell differentiation.

An "inducible" promoter is a promoter which is under more precise environmental or developmental control. Examples of environmental conditions that may effect transcription by inducible promoters include anaerobic conditions or the presence of light. Examples of promoters under developmental control include promoters that initiate transcription only in certain tissues, such as root specific promoters.

The term "anti-sense orientation" refers to the orientation of nucleic acid sequence from a structural gene that is inserted in an expression cassette in an inverted manner with respect to its naturally occurring orientation. When the sequence is double stranded, the strand that is the template strand in the naturally occurring orientation becomes the coding strand, and vice versa.

The term "uninterrupted" refers to a DNA sequence (e.g., cDNA) containing an open reading frame that lacks intervening, untranslated sequences.

The term "operably linked" refers to functional linkage between a promoter and a second sequence, wherein the promoter sequence initiates transcription of RNA corresponding to the second sequence.

A "heterologous sequence" or "heterologous expression cassette" is one that originates from a foreign species, or, if from the same species, is substantially modified from its original form.

WHAT IS CLAIMED IS:

1. A method of reducing nicotine levels in a plant, the method comprising introducing into the plant an expression cassette comprising a promoter sequence operably linked to a nucleic acid segment from a gene encoding an enzyme in the nicotine biosynthetic pathway.

2. The method of claim 1, wherein the plant is a member of the genus *Nicotiana*.

3. The method of claim 1, wherein the nucleic acid segment is linked to the promoter sequence in an anti-sense orientation.

4. The method of claim 1, wherein the nucleic acid segment comprises a full length coding region of the gene.

5. The method of claim 1, wherein the nucleic acid segment is uninterrupted.

6. The method of claim 1, wherein the gene encodes nicotine synthase.

7. The method of claim 1, wherein the promoter is constitutive.

8. The method of claim 1, wherein the expression cassette is introduced into the plant using *Agrobacterium*.

9. The method of claim 1, wherein the expression cassette is introduced into the plant using a sexual cross.

10. A method of reducing nicotine levels in a plant, the method comprising introducing into the plant an expression cassette comprising a promoter sequence operably linked to a nucleic acid segment from a gene encoding a nicotine degradative enzyme.

11. The method of claim 10, wherein the gene encodes nicotine demethylase.

12. The method of claim 10, wherein the gene is a bacterial gene.

13. The method of claim 10, wherein the promoter sequence is a root specific promoter.

14. The method of claim 10, wherein the nucleic acid segment comprises a full length coding region of the gene.

15. The method of claim 10, wherein the nucleic acid segment is uninterrupted.

16. The method of claim 10, wherein the plant is a member of the genus *Nicotiana*.

17. A plant comprising a heterologous expression cassette comprising a promoter sequence operably linked to a nucleic acid segment from a gene encoding an enzyme in the nicotine biosynthetic pathway.

18. The plant of claim 17, wherein the plant is a member of the genus *Nicotiana*.

19. The plant of claim 17, wherein the nucleic acid segment is linked to the promoter sequence in an anti-sense orientation.

20. The plant of claim 17, wherein the nucleic acid segment comprises a full length coding region of the gene.

21. The plant of claim 17, wherein the nucleic acid segment is uninterrupted.

22. The plant of claim 17, wherein the gene encodes nicotine synthase.

23. A plant comprising a heterologous expression cassette comprising a promoter sequence operably linked to a nucleic acid segment from a gene encoding a nicotine degradative enzyme.

24. The plant of claim 24, wherein the gene encodes nicotine demethylase.

25. The plant of claim 24 wherein the gene is a bacterial gene.

26. The plant of claim 24, wherein the promoter sequence is a root specific promoter.

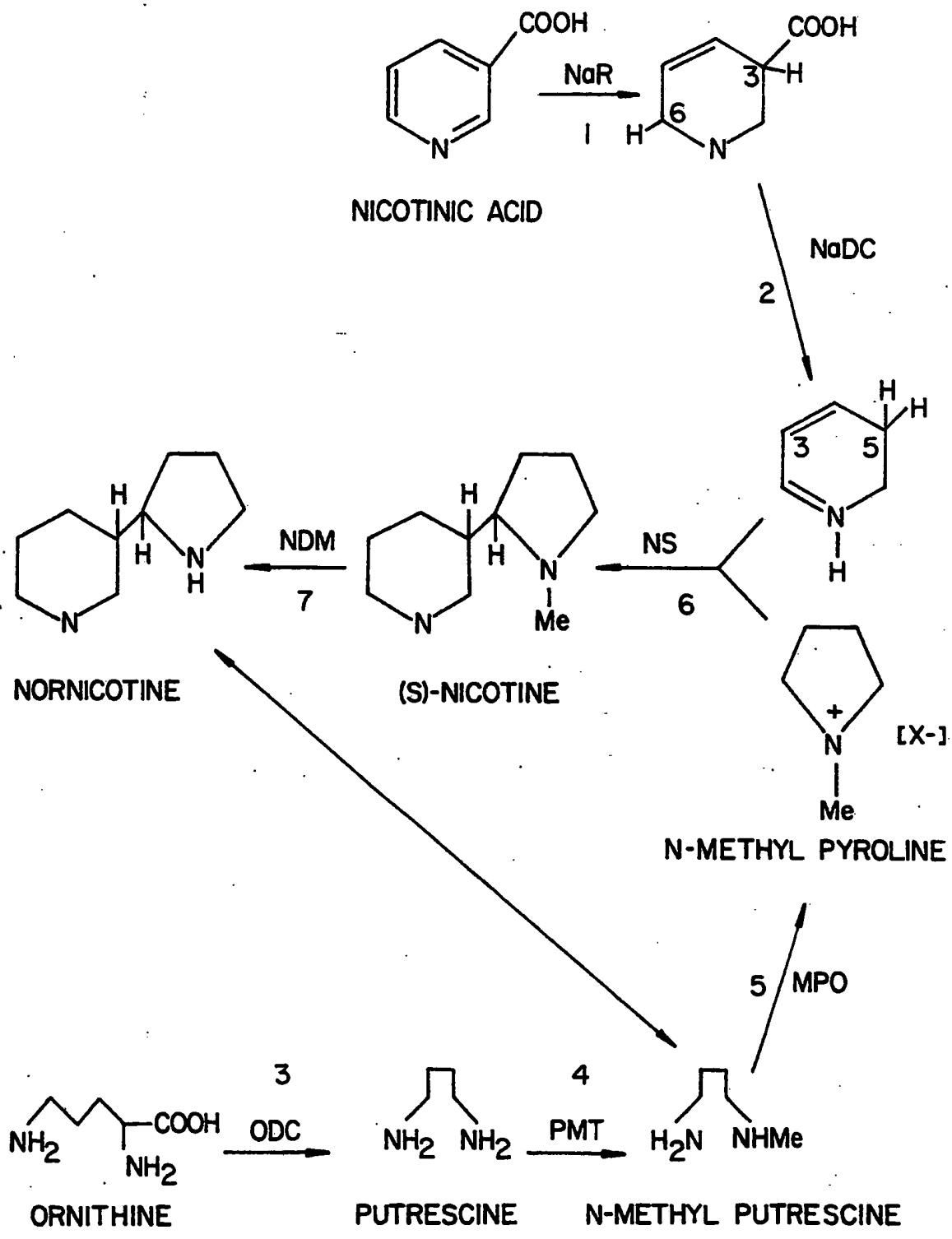
27. The plant of claim 24, wherein the nucleic acid segment comprises a full length coding region of the gene.

28. The plant of claim 24, wherein the nucleic acid segment is uninterrupted.

29. An expression cassette comprising a promoter sequence operably linked to nucleic acid segment from a gene encoding an enzyme in the nicotine biosynthetic pathway.

30. The expression cassette of claim 30 wherein the gene encodes nicotine synthase.

31. The expression cassette of claim 30 wherein the nucleic acid segment is linked to the promoter sequence in an anti-sense orientation.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3
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6
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N
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NaDC
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H
3
5
N
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NDM
7
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ORNITHINE
PUTRESCINE
N-METHYL PUTRESCINE

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/07752

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A01H 5/00
US CL :800/205; 435/172.3; 536/27
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/205, 200; 935/30; 435/172.3; 536/27

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Tetrahedron Letters, Volume 31, No. 44, issued 1990, Friesen et al, "Nicotine Synthase - An Enzyme from Nicotiana Species which Catalyzes the Formation of (S)-Nicotine from Nicotinic Acid and 1-Methyl-Pyrrolinium Chloride", pages 6295-6298, see pages 6296-6297.	6,22,30-31
A	Plant Molecular Biology, Vol. 15, issued July 1990, Hamill et al, "Over-expressing a Yeast Ornithine Decarboxylase Gene in Transgenic Roots of Nicotiana rustica can Lead to Enhanced Nicotine Accumulation", pages 27-38, see page 36.	1-5,7-9,17-21,29
A	Journal of Organic Chemistry, Vol. 41, No. 21, issued 1976, E. Leete, "A New Systematic Degradation of Nicotine to Determine Activity at C-2' and C-5'. The Pattern of Labeling in Nicotine and Nornicotine Formed from [2-^{14}C] Ornithine in Nicotiana glutinosa, and in Nicotine obtained from N. tabacum Exposed to [^{14}C, ^{13}C] Carbon Dioxide", pages 3438-3441, see entire document.	10-16,23-28

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
20 October 1992	02 DEC 1992
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer ELIZABETH F. MCELWAIN, PHD
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/07752

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NATURE, Vol. 334, issued 25 August 1988, C.J.S. Smith et al, "Antisense RNA Inhibition of Polygalacturonase Gene Expression in Transgenic Tomatoes", pages 724-726, see entire document.	3,19,31
A	Plant Physiology, Vol. 93, issued 1990, Conkling et al, "Isolation of Transcriptionally Regulated Root-Specific Genes from Tobacco", pages 1203-1211, see pages 1208-1209.	26